The Biophysics of Leaf Growth in Salt-Stressed Barley. A Study at the Cell Level[1]

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Biophysical parameters potentially involved in growth regulation were studied at the single-cell level in the third leaf of barley (*Hordeum vulgare*) after exposure to various degrees of NaCl stress for 3 to 5 d. Gradients of elongation growth were measured, and turgor pressure, osmolality, and water potentials (ψ) were determined (pressure probe and picoliter osmometry) in epidermal cells of the elongation zone and the mature blade. Cells in the elongation zone adjusted to decreasing external ψ through increases in cell osmolality that were accomplished by increased solute loads and reduced water contents. Cell turgor changed only slightly. In contrast, decreases in turgor also contributed significantly to ψ adjustment in the mature blade. Solute deposition rates in the elongation zone increased at moderate stress levels as compared with control conditions, but decreased again at more severe NaCl exposure. Growth-associated ψ gradients between expanding epidermal cells and the xylem were significant under control and moderate stress conditions (75 mM NaCl) but seemed negligible at severe stress (120 mM NaCl). We conclude that leaf cell elongation in NaCl-treated barley is probably limited by the rate at which solutes can be taken up to generate turgor, particularly at high NaCl levels.

Salt stress causes a rapid and potentially lasting reduction in the rate of leaf growth (Munns, 1993). A reduction of the velocity of leaf elongation results from a reduction in the number of elongating cells or a reduction in the rate of cell elongation or from both. From the biophysical point of view (Cosgrove, 1993), a leaf cell of a NaCl-treated plant can expand at reduced rates because of reduced uptake rates of water or osmolytes, because of hardened walls, or because of lowered turgor. These stress effects are based on four mechanisms. First, osmotically driven uptake of water, which is necessary for cell enlargement, may be inhibited by low water potentials (ψ) in the root space (osmotic stress). Second, specific solutes normally used to generate osmotic pressure may not be available at sufficient quantities because of competition by Na^+ or Cl^- for uptake (nutrient imbalance). Third, even if external Na^+ and Cl^- provide a sufficient source of osmolytes, cells may not be able to cope with these adequately and may eventually suffer from toxic effects (ion toxicity). Fourth, cells may produce specific reactions to elevated NaCl, e.g. altered rates of wall synthesis (regulatory response).

With two exceptions (Thiel et al., 1988; Arif and Tomos, 1993), previous studies on the biophysical control of leaf elongation in NaCl-stressed grass leaves have been carried out at the bulk tissue rather than at the cell level. These studies concluded that cell turgor was unchanged in response to NaCl and that altered wall properties, altered apoplastic solute concentrations, or non-biophysical causes were responsible for the reduction in leaf elongation (Matsuda and Riazi, 1981; Termaat et al., 1985; Thiel et al., 1988; Arif and Tomos, 1993; Munns, 1993).

The aim of the present study was to relate at the cellular level changes in biophysical parameters to changes in leaf elongation velocity caused by NaCl. Cell turgor was measured with the cell-pressure probe in planta, osmolality was determined by picoliter osmometry of extracted cell sap, and cell ψ was derived from turgor and osmolality data. In addition, osmolality and water content were analyzed at bulk-tissue level, and relative elemental growth rates (REGR) were determined along the elongation zone. These measurements allowed calculation of deposition rates of osmolytes along the growth zone and the distinction between various factors contributing to osmotic and ψ adjustment in elongating and mature cells. Because grass leaf growth zones are enclosed by the sheaths of older leaves, destructive preparation was unavoidable to gain access to the basal region of leaf three, the first leaf considered to be entirely dependent on external or photosynthetic nutrient supply. Three different preparation techniques were pursued and compared in their effects on cell turgor, osmolality, and growth-associated ψ gradients.

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RESULTS

NaCl Effects on Leaf and Cell Elongation Growth in Planta

NaCl in the root medium inhibited the elongation velocities of third leaves of barley (*Hordeum vulgare*) plants. Between batches of plants, means of maximal elongation velocities ranged from 2.57 to 2.98 mm h^{-1} (control), from 2.09 to 2.53 mm h^{-1} (75 mM NaCl), and from 1.79 to 2.12 mm h^{-1} (120 mM NaCl), respectively (not shown). Elongation growth was detected between 4 and 50 mm above the leaf insertion by pin-prick experiments in plants at all stress levels (control and 75 and 120 mM NaCl; Fig. 1). In contrast, maximum rates of relative elemental growth (REGR) decreased with increasing NaCl concentrations. With increasing salt stress, the bell-shaped REGR profile observed in control plants (Fig. 1A) became flatter, and the dominant peak vanished (Fig. 1, B and C). No NaCl-associated growth inhibition occurred within the first 7 to 8 mm above the leaf base. Growth was equally unaffected distal of 30 mm by 75 mM NaCl but was reduced in this region by 120 mM NaCl (Fig. 1D).

The effect of reduced leaf elongation velocities was partly compensated by an 0.5- to 2-d longer duration of leaf (three) elongation in NaCl plants (not shown). Final leaf and cell length was reduced by NaCl (not shown). Final leaf length was 34.8 ± 2.9 cm in control, 33.2 ± 2.7 cm in 75 mM NaCl, and 30.5 ± 4.7 cm in 120 mM NaCl plants (means ± SD of 10–17 plant analyses). Final length of near-stomatal cells in the adaxial epidermis at the time of maximum and steady leaf elongation velocity was 191 ± 30 μm in control, 161 ± 13 μm in 75 mM NaCl, and 158 ± 13 μm in 120 mM NaCl plants (means ± SD of four plants, with 19 cell analyses each). Epidermal cell flux through the elongation zone was 15.7 ± 2.3, 13.4 ± 1.6, and 13.3 ± 0.6 cells (cell file)$^{-1}$ h^{-1} in control, 75 mM NaCl-, and 120 mM NaCl-treated plants, respectively. Differences in cell length and cell flux between treatments were not significant. Compa-

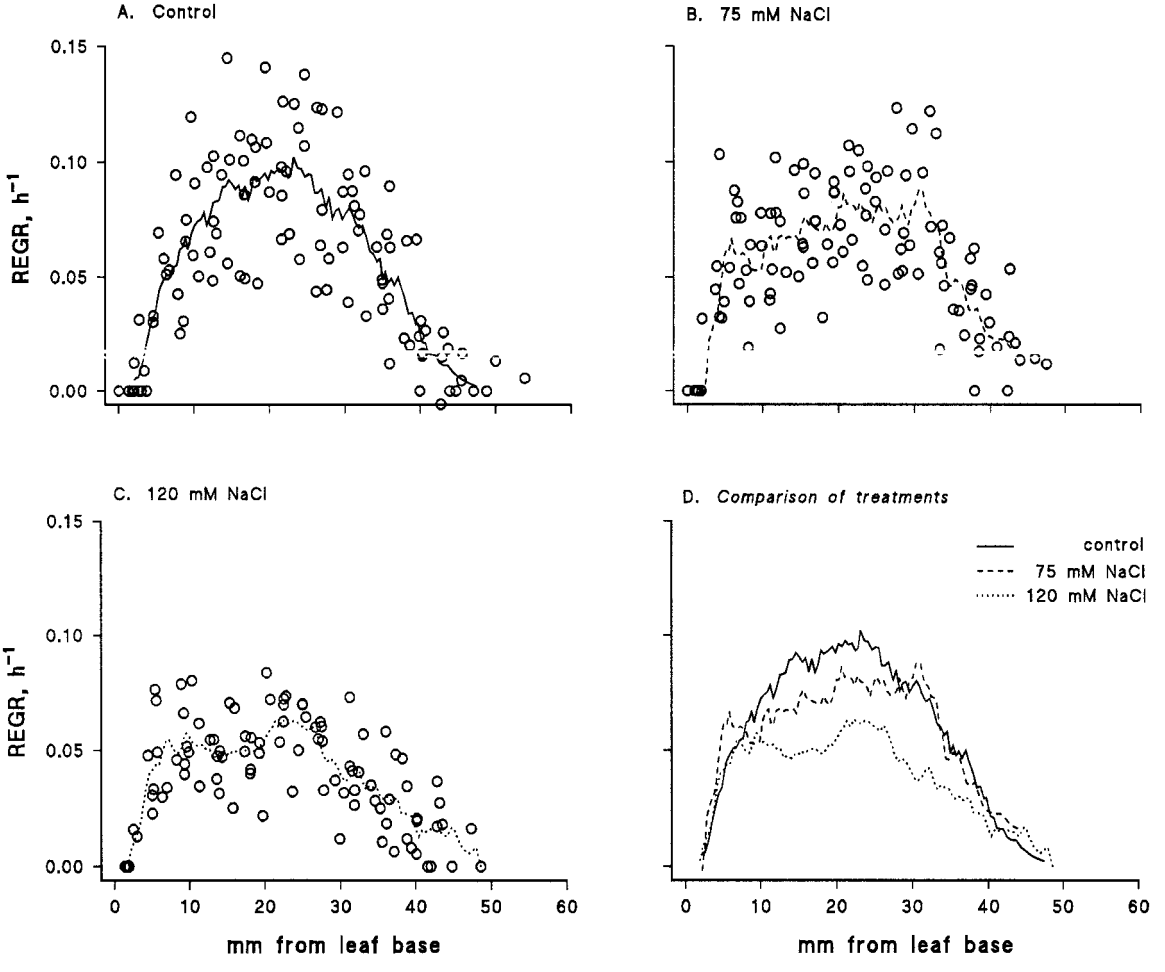

Figure 1. Profiles of REGR along the basal region of leaf three of barley grown under control conditions (A) or grown on nutrient solution containing 75 mM (B) or 120 mM NaCl (C). In A, B, and C, original data from pin-prick experiments performed in 10 to 11 individual plants are given together with a curve showing the running mean of nine consecutive data points. For comparison, D shows curves without original data.

rable reduction in final cell and leaf length in NaCl plants indicates that the total number of epidermal cells per cell file and leaf (blade) changed little and that NaCl had no significant effect on overall cell production.

Leaf Elongation on the Probe Stage

To gain access to cells in the basal region of the third leaf for measuring turgor and single-cell osmolality, three alternative methods were employed (see "Materials and Methods" for details). Methods I and II involved removal of the older leaves and lining the exposed third leaf base with tissue paper soaked in distilled water (method I) or in a NaCl solution of the concentration present in the root medium (method II). Method III was less destructive, because only the oldest leaf was removed, followed by cutting a window into the sheath of leaf two. To evaluate the effect of the preparation and transfer to the probe stage, leaf elongation velocity was measured on plants mounted in the experimental setup.

The method of preparation did affect leaf elongation (Table I). In plants prepared according to method I, the NaCl-dependent growth inhibition observed in undisturbed plants was not maintained on the probe stage. Leaves of NaCl-treated individuals elongated faster than the control, although leaf elongation generally proceeded 39% to 60% slower than in undisturbed plants. In method II, leaf elongation growth was reduced even more, by 77% to 82%. When only a small window was cut into the sheath of leaf 2 (method III), leaf elongation velocity was inhibited by 47% to 54% before plants were transferred to the probe stage. Mounting the plants on the stage slowed leaf growth even further.

Table I. *Elongation velocities of third leaves of barley plants following various treatments*

Leaf elongation velocities (means ± SD of three to 11 leaves analyzed) are listed for plants exposed to three levels of NaCl in the root medium (control, i.e. 1, 75, and 120 mM), before and after preparation according to three alternative methods (see "Materials and Methods" for full details) and transfer to the probe stage. For method III, elongation velocities at an intermediate state of preparation ("window cut") are also given. n.a., Not available.

Preparation Method and State of Plant	Control	75 mM NaCl	120 mM NaCl
		mm h⁻¹	
Method I			
Undisturbed	2.90 ± 0.45	2.49 ± 0.42	2.12 ± 0.29
On probe stage	1.15 ± 0.33	1.37 ± 0.96	1.30 ± 0.56
Method II			
Undisturbed	2.98 ± 0.28	2.10 ± 0.40	1.98 ± 0.26
On probe stage	n.a.	0.48 ± 0.41	0.35 ± 0.26
Method III			
Undisturbed	2.70 ± 0.27	2.09 ± 0.19	1.90 ± 0.22
Window cut	1.42 ± 0.25	0.96 ± 0.13	0.87 ± 0.22
On probe stage	0.94 ± 0.25	0.53 ± 0.12	0.61 ± 0.09

Epidermal Cell Turgor

Epidermal cell turgor was measured at three locations along the basal leaf region (20, 40, and 60 mm, corresponding to zones of rapid, slow, and no elongation growth, respectively) and in the mature blade between 6 to 8 cm above the ligule of leaf 2.

In plants prepared according to method I, turgor seemed uniform in the elongation zone (20 and 40 mm) and increased beyond it (60 mm; Fig. 2A). Turgor generally was lowest in control plants (although not always significant statistically, Fig. 2A). In NaCl-treated method II plants (treatment of control plants prepared by this method would have differed insignificantly from that of control plants of method I), a gradual increase of turgor with increasing distance from the leaf base was observed (Fig. 2B). In general, turgor was lower in method II than in method I. In plants prepared according to method III, turgor was measured only at the position of the window in the older leaf sheath (20–24 mm from the leaf base); it was practically identical in all treatments (Fig. 2C). In the emerged, air-exposed leaf blade, turgor was always higher than in actively growing cells and decreased significantly with increasing salt stress (Fig. 2D). The suitability for turgor analysis of preparation methods I through III and the possibility of artifacts is discussed further below.

Osmolality in Epidermal Cells and Bulk-Leaf Extracts

Single-cell osmolality was analyzed at five locations in the basal region (10, 20, 30, 40, and 60 mm) of third leaves of plants prepared according to methods I and II. It did not seem necessary to determine single-cell osmolality in method III plants, because this method was the least intrusive and because methods I and II presented extremes of tissue handling (moistening with either distilled water or NaCl solutions). Single-cell osmolality was also determined in the emerged blade of leaf three (6–8 cm above the ligule of leaf two); samples were taken from the same leaves in which turgor had been measured (see above).

Osmolality of epidermal cells generally was uniform along basal leaf zones, and it increased with increasing NaCl exposure (Fig. 3, A and B). Epidermal cell osmolality in the mature part of the leaf blade was identical to values in the basal region in NaCl-treated plants, but it was significantly higher than basal region osmolalities in control plants (Fig. 3C). As in cells of the basal region, cell osmolality increased with NaCl stress.

Osmolality was also measured in bulk-leaf extracts from three segments of the basal leaf region (0–25, 25–50, and 50–60 mm). No significant differences between the three locations were detected in any treatment (Fig. 3D). Bulk osmolality and epidermal cell osmolality did not differ significantly along the leaf basis, suggesting that cell osmolalities and NaCl

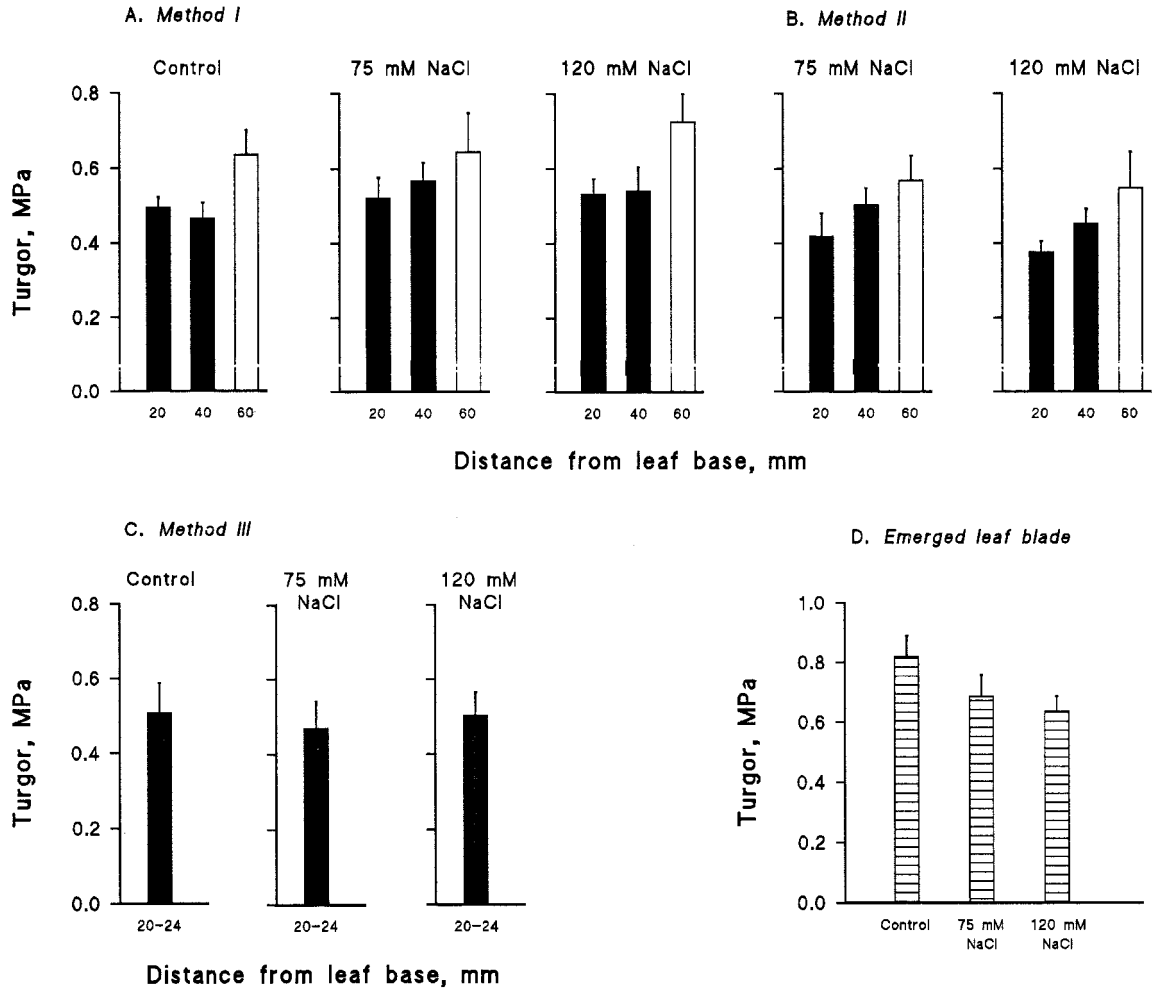

Figure 2. Epidermal cell turgor pressure along the basal region (A–C) and in the emerged part (D) of leaf three of barley grown under control conditions or on nutrient solution containing 75 or 120 mM NaCl as indicated. The base of the third leaf was made accessible by one of three preparation methods (A, B, and C, respectively; for methodical details see "Materials and Methods"). Turgor values measured in basal region cells that were actively growing are given as black bars, whereas values from cells that had ceased to elongate are symbolized by white bars (in A–C; compare with Fig. 1). Mature cells (D; hatched bars) had emerged from the older leaf sheaths more than 1 d before the experiment. Turgor was measured with the cell-pressure probe in five to six plants of each treatment, with three to six cells measured at each location. Values given are means ± SD. NaCl-treated plants in A and B showed at various positions statistically significant ($P < 0.05$ in Student's *t* test) differences in turgor as compared with turgor values in control plants (A, at 40 mm for 75 mM NaCl, and at all positions for 120 mM NaCl; B, at 20 mm for 75 mM and for 120 mM NaCl). Along the leaf region enclosed by older sheaths, turgor was always significantly higher outside of the elongation zone (white bars in A ands B) than within it (black bars in A and B). In the emerged blade (D), cell turgor was significantly higher in the control than in the NaCl-treated plants; the difference between NaCl treatments was not significant in D.

responses were similar in the epidermis and other leaf tissues.

ψ and Growth-Associated ψ Gradients

Cell ψ was calculated from osmolality and turgor data. Whereas turgor data had been obtained using all preparation methods, osmolality data were available only for methods I and II. Both methods, which represented extremes of tissue handling as discussed above, gave almost identical osmolalities. Therefore, it seemed justified to average osmolality values from methods I and II and use it to calculate ψ for method III plants.

In the basal leaf region, epidermal cell ψ decreased in parallel with the decrease of root medium ψ under salt stress conditions (Fig. 4, A–C). ψ of growing cells (20 and 40 mm above the leaf insertion; compare Fig. 1) were always more negative than ψ of cells that had ceased to elongate (Fig. 4, A and B). Differences in ψ between cells that elongated at high (20 mm) or low (40 mm) relative rates were less consistent. In NaCl-treated plants, the method of preparation affected ψ. Method II (exposed leaf base in contact with NaCl

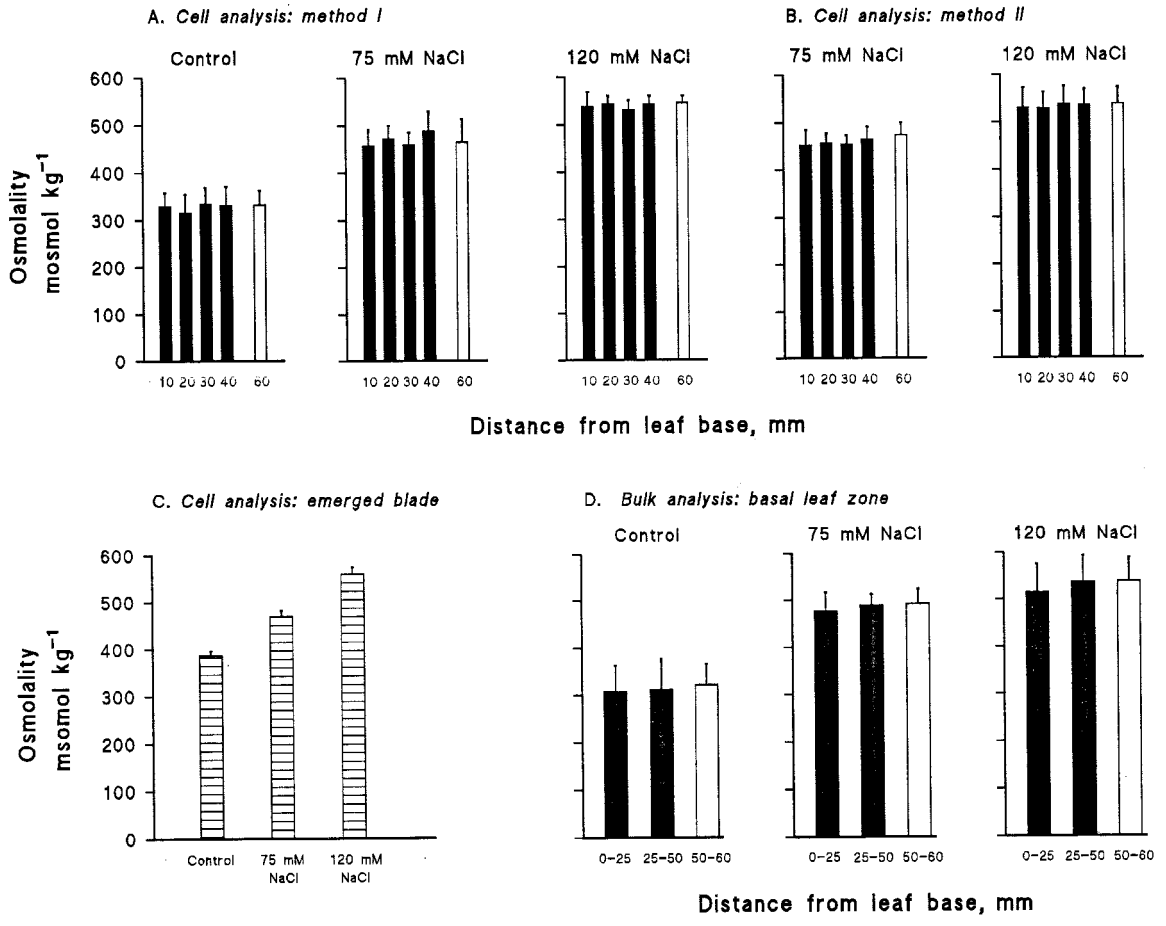

Figure 3. Osmolality in epidermal cells (A–C) and bulk-leaf extracts (D) of the third leaf of barley grown under control conditions or grown on nutrient solution containing 75 or 120 mM NaCl as indicated. Cell osmolality was determined by picoliter osmometry of cell sap extracted at five different locations along the basal leaf region (A and B) or halfway along the emerged part of the blade (C). Cells of the basal region were made accessible by either of two methods (A and B; see "Materials and Methods" for full details). Data obtained from cells located within the elongation zone are marked by black bars, whereas data from cells that had ceased to elongate are given by white bars. In the emerged part of the leaf (hatched bars), cells were fully mature and had been exposed to the atmosphere for more than 1 d. Results are means ± SD of five to seven leaf analyses.

solution; Fig. 4B) yielded the most negative cell ψ (and lowest turgor; compare Fig. 2). In contrast, method I (leaf base in contact with distilled water; Fig. 4A) gave the least negative cell ψ (and highest turgor; compare Fig. 2) for NaCl plants. Method III yielded intermediate ψ (Fig. 4C). In control plants, methods I and III gave almost identical cell ψ (at 20 mm; Fig. 4, A and C). Epidermal cell ψ in the expanded blade was obtained from osmolality and turgor measurements carried out on identical leaves as described above. ψ in mature cells decreased with increasing salt levels but tended to be less negative than in the basal leaf region (Fig. 4D).

Because cell expansion may be limited by tissue water transport properties, we attempted to quantify growth-associated ψ gradients between expanding leaf cells and leaf xylem. Equipment for measuring xylem ψ directly was not available. Instead, we used ψ determined in epidermal cells of the transpiring mature leaf blade as a most negative possible estimate of xylem ψ. In all but one of the locations investigated along the basal leaf region, growth-associated ψ gradients were found, regardless of treatment and method of preparation (Fig. 5). In one case (60 mm, 120 mM NaCl, method I; Fig. 5A), the ψ gradient was positive, i.e. driving water toward the xylem. This unexpected result may have been due to either an artifact caused by the preparation method (increased turgor in tissue that was in contact with distilled water) or xylem ψ being considerably less negative than suggested by epidermal cell ψ in the expanded blade.

ψ gradients were significantly larger at 20 and 40 mm, i.e. within the elongation zone, than at 60 mm, just outside the growth zone (Fig. 5, A and B). For any given preparation method, growth-associated ψ gradients in the elongation zone were smallest in plants exposed to 120 mM NaCl. Thus, growth-

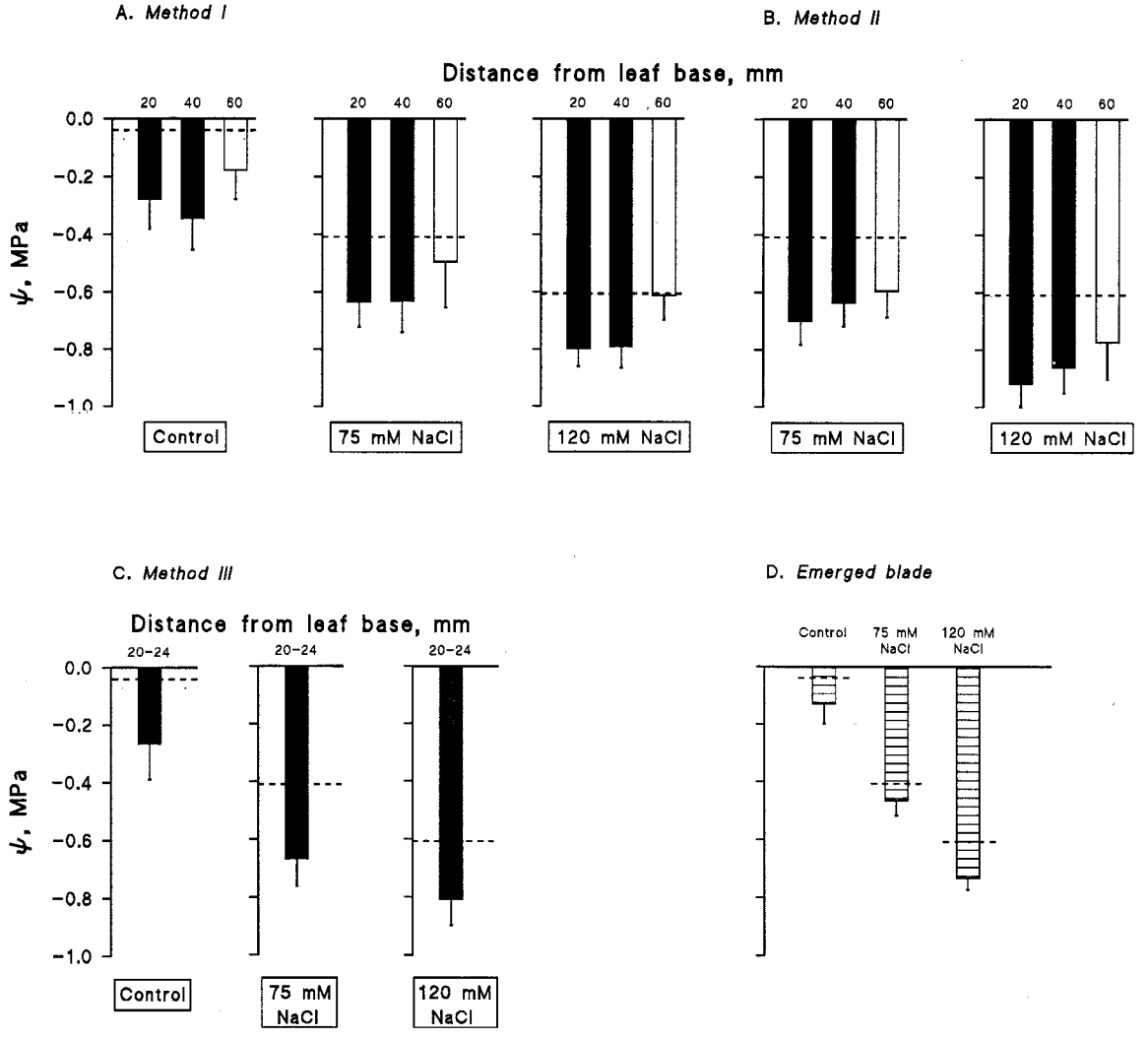

Figure 4. ψ of epidermal cells along the basal region (A–C) and the emerged part (D) of leaf three of barley grown under control conditions, or grown on nutrient solution containing 75 or 120 mM NaCl as indicated. Growth media ψ are given as dashed lines. ψ of cells were calculated from cell turgor (see Fig. 2) and cell osmolality (see Fig. 3). Data obtained from cells located within the elongation zone are marked by black bars, whereas data from cells that had ceased to elongate are given by white bars. In the emerged part of the leaf (hatched bars), cells were fully mature and had been exposed to the atmosphere for more than 1 d.

associated ψ gradients tended to be larger at locations where elongation growth occurred and to be smaller when growth was severely inhibited by salt stress. However, no clear relation between the magnitude of ψ gradients and growth became apparent when control and 75 mM NaCl plants were compared (Fig. 5).

Osmotic Adjustment

The adjustment of cell ψ after changes in external ψ can be achieved by various means. By definition (Barlow, 1986), osmotic adjustment is a way of adjusting ψ that is accomplished (a) without change in turgor and (b) through net solute accumulation rather than a decrease in water content. As Figure 6 shows, epidermal cells throughout the leaf adjusted cell ψ to closely match the changes in external ψ imposed by addition of NaCl. Mature cells adjusted ψ through changes in both osmolality and turgor, whereas the contribution of turgor was negligible in the basal leaf region. Thus, only basally located cells satisfy the first criterion for osmotic adjustment.

To test whether the tissues at the leaf base also fulfilled the second criterion, the basal leaf region was analyzed for water contents. In control and stressed plants, water content per unit of leaf length increased significantly along the basal leaf region, even beyond the zone of elongation growth (Fig. 7). Therefore, cells must have continuously expanded radially and/or tangentially as they moved along the elongation zone and beyond. Bulk water content per unit of leaf length was generally lower in stressed than in control plants (Fig. 7). Water content and osmolality data allowed the calculation of the total

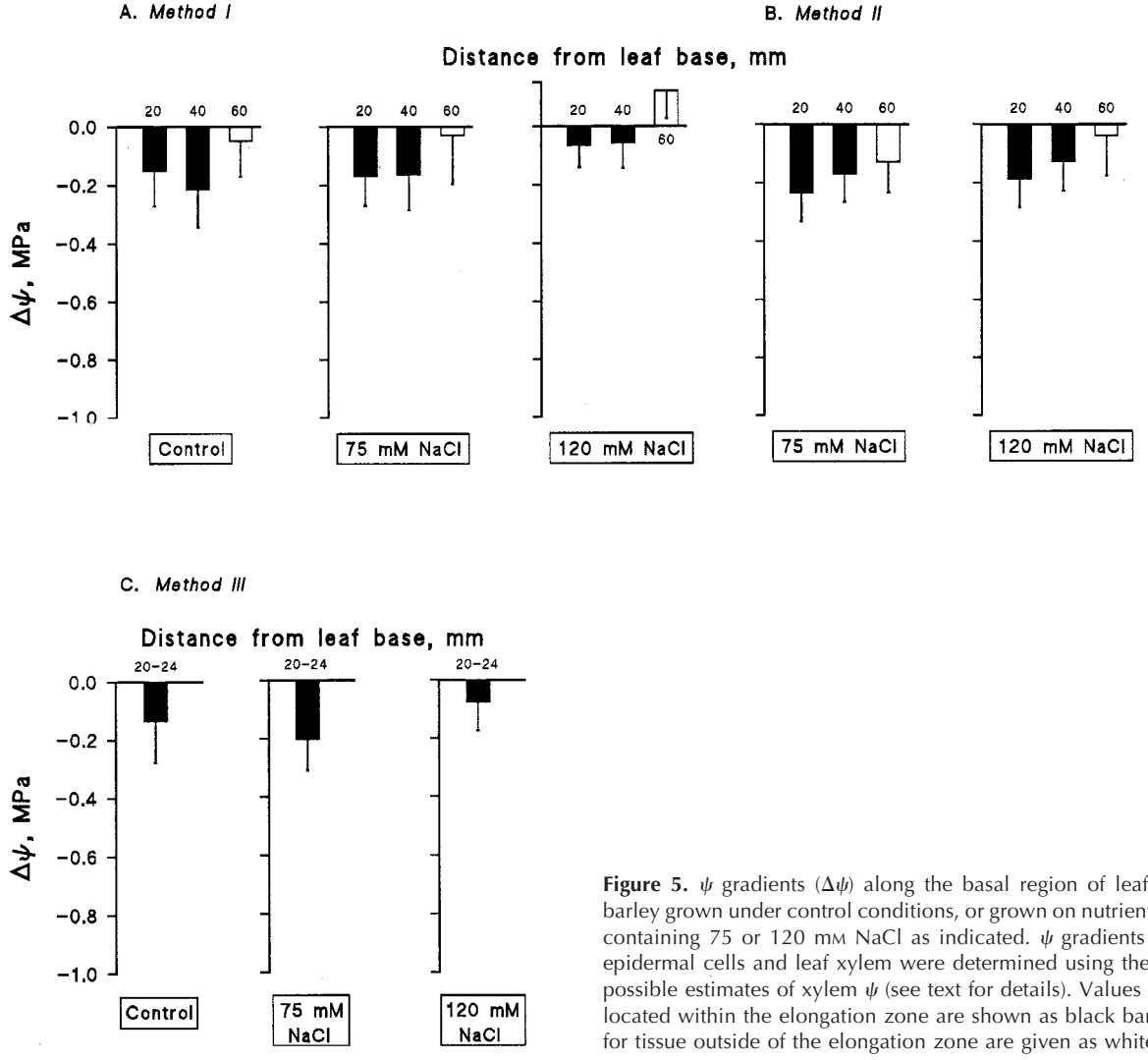

Figure 5. ψ gradients ($\Delta\psi$) along the basal region of leaf three of barley grown under control conditions, or grown on nutrient solution containing 75 or 120 mM NaCl as indicated. ψ gradients between epidermal cells and leaf xylem were determined using the smallest possible estimates of xylem ψ (see text for details). Values for tissue located within the elongation zone are shown as black bars; values for tissue outside of the elongation zone are given as white bars.

amount of osmotically active solutes per unit of leaf length. Exposure of plants to 75 mM NaCl caused a uniform increase (24%–28%) in solutes throughout the basal leaf region compared with the control (Table II). No significant increase in the total solute load per unit of leaf length could be evoked by increasing the external NaCl concentration from 75 to 120 mM (Table II). The increased solute load along the basal leaf region of NaCl-treated plants accounted only for about one-half of the increase in osmolality under salt stress (Table III). Thus, decreases in water content played a significant role in the adjustment of cell ψ. Assuming that water contents of epidermal cells along the basal leaf region changed in a way similar to that at bulk-leaf level, it can be concluded that the cells did not fulfill the second criterion for osmotic adjustment.

Deposition Rates of Solutes in the Basal Leaf Region

The amount of solutes deposited per time in the elongation zone of leaf three increased at moderate salt stress (75 mM NaCl) but decreased again at higher NaCl levels (120 mM) in the root medium (Fig. 8A). Expressed on a tissue-water basis, deposition rates of solutes increased at both NaCl concentrations, particularly at 75 mM NaCl (Fig. 8B). The deposition rate at 75 mM NaCl corresponded to an average of 0.06 MPa of turgor generated per hour along the elongation zone and across all tissues.

Transpiration Rates of Plants

At the time of analyses, transpirational water loss of leaf three was 212 (two independent determinations: 240 and 183), 141 (151 and 131) and 130 (130 and 130) μL leaf^{-1} h^{-1} for control, 75 mM NaCl, and 120 mM NaCl plants, respectively.

DISCUSSION

The present study provides the first complete set of the biophysical key parameters—turgor, osmolality, and ψ—for single cells of leaves growing under salt

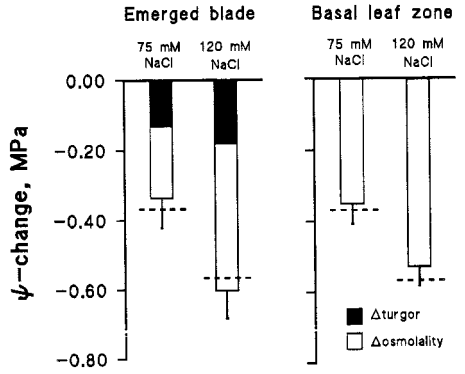

Figure 6. Contribution of changes in turgor and changes in osmolality to the adjustment of epidermal cell ψ in response to changes in root medium ψ. Adjustment was studied in the emerged part and in the basal region of leaf three. Changes in cell ψ are given relative to the situation in control experiments, where the nutrient medium ψ was −0.4 MPa. Stressed plants were grown on media containing 75 or 120 mM NaCl (as indicated); the corresponding decrease in external ψ is indicated by dashed lines. To simplify presentation of data for the basal leaf region, values obtained at 20, 40, and 60 mm from the leaf base were pooled (compare Figs. 2–4) and averaged. The contribution of turgor changes to ψ adjustment along the basal leaf zone was negligible (<1%) and does not show in the graph.

stress. Two previous studies reported single-cell turgor measurements on NaCl-stressed cereal leaves (barley [Thiel et al., 1988] and wheat [*Triticum aestivum*; Arif and Tomos, 1993]). However, these studies focused on transient turgor changes in response to the addition of NaCl to the nutrient solution, whereas we were concerned with longer-term effects of salt stress on leaf growth. Exposure to NaCl started 1 to 2 d before the emergence of leaf three, which was analyzed 4 to 6 d later when it was elongating at a steady and maximum velocity (Fricke et al., 1997).

Growth Profiles and NaCl Stress

Treatment of barley with 75 or 120 mM NaCl reduced maximum rates of REGR in leaf three but did not affect the length of the elongation zone (Fig. 1). Similar results were obtained for the same cultivar subjected to nitrogen-limitation (Fricke et al., 1997) and for leaves three, four, and five of wheat exposed to three NaCl levels (Hu et al., 2000). In contrast, a reduction in both maximal REGR and length of the leaf elongation zone has been observed in sorghum (*Sorghum bicolor*) treated with 100 mM NaCl (Bernstein et al., 1993) and in maize (*Zea mays*) treated with 80 mM NaCl (W. Fricke and W.S. Peters, unpublished data). Thus, members of the Poaceae subfamilies Panicoideae (including *Zea* spp. and *Sorghum* spp.) and Pooideae (including *Triticum* spp. and *Hordeum* spp.) seem to respond differently to growth-reducing stress.

In our study, salt-induced growth inhibition acted by limiting maximum relative rates of cell expansion while leaving cells growing at lower rates unaffected. Thus, NaCl did not reduce leaf growth through causes (e.g. ion toxicity) that induced general, percentage-type inhibitions of cellular activities. Munns et al. (1982) reached a similar conclusion when studying bulk ion and carbohydrate concentration along the growth zone of leaf three of NaCl-treated barley.

Effects of Preparation Techniques

Cell osmolality was not affected by the preparation method (I and II). Moreover, osmolality of cells was similar to osmolality in bulk-leaf extracts from undisturbed plants. We conclude that our osmolality data from single cells reflect the situation in situ. This is further supported by recent results, which show that cell osmolality is the same for plants prepared according to method I or III (W. Fricke, unpublished data).

Turgor and ψ data (Figs. 2 and 4) suggest that method I, which included covering exposed leaf tissue with tissue paper soaked in distilled water, caused a relief of water stress, whereas method II, which had the tissue paper soaked in NaCl solution, caused an additional water stress in NaCl plants. Turgor, cell ψ, and leaf elongation are known to respond within seconds to minutes to changes in external ψ (Acevedo et al., 1971; Cutler et al., 1980; Thiel et al., 1988; Arif and Tomos, 1993). Method III, which involved cutting a window into the sheath of leaf two, was the least intrusive method. This method previously had been employed successfully to measure turgor along the basal region of elongating leaves of English ryegrass (*Lolium temulentum*; Thomas et al., 1989), barley (Pollock et al., 1990), tall fescue (*Festuca arundinacea*; Martre et al., 1999), and maize seedlings (Thompson et al., 1997). For NaCl plants, turgor obtained by method III was intermediate between values obtained by methods I and II. In control plants, turgor was almost identical to that obtained by method I (method II not employed; Fig. 2). It seems that method III yielded turgor values resembling most those in undisturbed plants.

The above considerations do not prove that turgor obtained through method III was unaffected by the preparation technique. It could be argued that method III reduced leaf elongation velocity and, as a result, cell turgor and ψ—hence, the appearance of substantial growth-associated ψ gradients. We cannot rule out this possibility, but we consider it unlikely, for two major reasons.

Only a handful of studies exist in which cell turgor has been measured in the elongation zone of grass leaves. In each case, turgor was measured with the micropressure probe, and the preparation technique affected leaf elongation velocity. However, despite large variation in residual elongation velocity (after the plant preparation for turgor analyses) and a wide

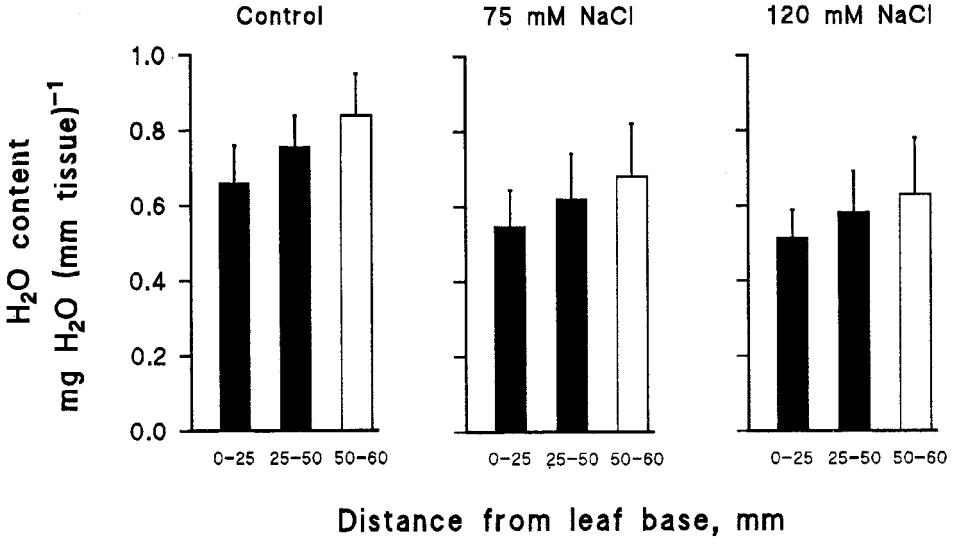

Figure 7. Water content per unit of leaf length along the basal region of leaf three of barley grown under control conditions, or grown on nutrient solution containing 75 or 120 mM NaCl as indicated. Black bars represent data from tissue located within the elongation zone (compare Fig. 1). The continuing increase of water content beyond the elongation zone indicates substantial leaf growth in width and/or diameter in the absence of tissue elongation. Means ± SD of 12 leaves analyzed are shown.

range of elongation velocities of stress treatments, turgor was similar. Fricke et al. (1997) obtained turgor of 0.48 to 0.53 MPa for leaf three of barley grown under control-conditions or under two levels of nitrogen-limitation; reduction in leaf elongation velocity due to plant preparation (partial removal of older sheath) was approximately 50%. Arif and Tomos (1993) measured turgor in the first leaf of wheat after the addition of 25 or 150 mM NaCl to the root medium. The authors gained access to the elongation zone using the "window-cut" approach and observed that turgor remained between 0.45 to 0.50 MPa for the duration of the experiment (6 h). Thomas et al. (1989) used the same approach to study English ryegrass and analyzed plants with >90% residual leaf elongation velocity. Turgor was about 0.5 MPa. Pollock et al. (1990) measured turgor in the first leaf of barley, using the window-cut approach, and the authors considered only plants with >80% residual leaf elongation velocity. Despite a 10-fold difference in leaf elongation velocity due to changes in meristem temperature, turgor differed little and ranged from 0.63 to 0.68 MPa. Thompson et al. (1997) measured turgor in the first leaf of nutrient-sufficient maize and observed no changes in turgor after addition of PEG to the root medium. Turgor was between 0.5 to 0.6 MPa. Martre et al. (1999) analyzed turgor in tall fescue leaves by gaining access through the window-cut approach. The authors considered only plants with approximately 80% residual leaf elongation velocity and obtained a mean turgor of 0.53 ± 0.01 MPa.

Tomos et al. (1984) measured turgor in red beet (*Beta vulgaris*) storage tissue. The authors concluded that excision of tissue caused turgor to decrease substantially due to the accumulation of solutes leaked from damaged cells in the apoplast (which lowered cell ψ and turgor). In the present study, cells of the sheath of leaf two were damaged to gain access to leaf three. Increase in apoplastic solute concentration

Table II. *Total amount of osmotically active solutes along the basal region of leaf three of barley grown at different NaCl concentration in the root medium*

Bulk osmolality was multiplied with bulk water content to determine the total amount of osmotically active solutes. Possible deviations of osmotic coefficients from 1 were not considered. Therefore, values represent lowest possible estimates.

Salt Exposure	0 to 25 mm from Leaf Base	25 to 50 mm from Leaf Base	50 to 60 mm from Leaf Base
	μmol mm⁻¹ leaf length		
Control	0.204 ± 0.048	0.237 ± 0.055	0.270 ± 0.052
75 mM NaCl	0.262 ± 0.051	0.304 ± 0.060	0.335 ± 0.072
120 mM NaCl	0.266 ± 0.048	0.314 ± 0.066	0.339 ± 0.087

Table III. *The contribution of increased solute load to osmolality rises in bulk-leaf tissue of NaCl-treated barley*

NaCl-related increases in the total amount of osmotically active solutes along the basal region of leaf three were related to increases predicted from osmolality measured in bulk tissue extracts. The percentages of the NaCl-related increases in osmolality that were due to increases in the total solute load rather than decreases in water content are shown.

Salt Exposure	0 to 25 mm from Leaf Base	25 to 50 mm from Leaf Base	50 to 60 mm from Leaf Base
		%	
75 mM NaCl	51	49	45
120 mM NaCl	44	44	39

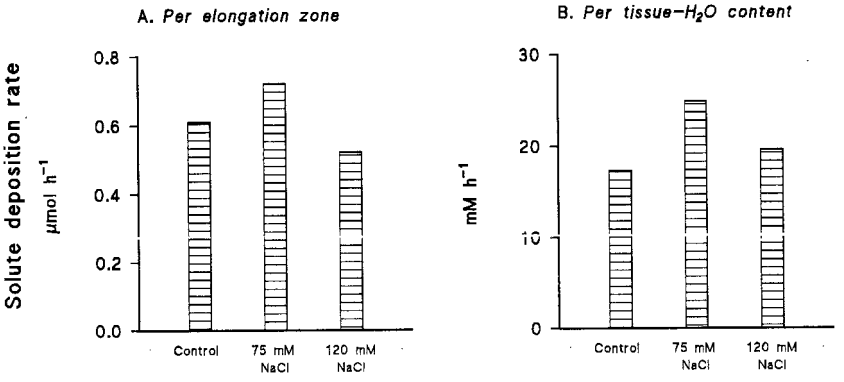

Figure 8. Deposition rates of solutes along the elongation zone of leaf three of barley grown under control conditions or grown on nutrient solution containing 75 or 120 mM NaCl as indicated. Deposition rates are expressed either as the amount of solutes deposited per elongation zone and hour (A) or as the amount of solutes deposited per water content of the elongation zone and hour (B).

may have reduced turgor (and ψ) in the underlying epidermal cells of leaf three. If so, one would have expected for control plants that turgor obtained through method III (window-cut approach) would be significantly lower than turgor in plants prepared according to method I. The latter method involved lining the exposed elongation zone with tissue soaked in distilled water, and this should have washed away or diluted apoplastic solutes. However, this was not the case. Turgor was 0.51 and 0.50 MPa, respectively. Meshcheryakov et al. (1992) observed in the hypocotyl of growing castor bean (*Ricinus communis*) seedlings that radial gradients in turgor across the cortex disappeared or reappeared when water supply was reduced or reestablished, respectively. The authors explained their results with a coupling of water and solute flows—in particular, differential changes in the storage capacity of apoplast and symplast. However, in tissues with a small apoplastic water volume, such as the present one, the storage capacity of the symplast should dampen changes in apoplastic solute concentrations.

Cell Turgor in the Elongation Zone of NaCl-Stressed Leaves

In general, turgor showed no clear relation to the velocity of leaf elongation of treatments (compare Fig. 2 and Table I). Neither was there any conceivable correlation between turgor at various positions on the leaf and the REGR profile in any treatment (compare Figs. 1 and 2). The results corroborate previous findings reported from NaCl-treated wheat (Arif and Tomos, 1993) and N-limited barley (Fricke et al., 1997) and confirm at cell level what Termaat et al. (1985) concluded from bulk-leaf and pressure bomb studies: Turgor does not limit growth in NaCl-treated barley and wheat. Similarly, Matsuda and Riazi (1981) observed that bulk-leaf turgor was largely unaffected in the basal leaf zone of osmotically stressed barley seedlings, and Michelena and Boyer (1982) reached the same conclusion for maize. Without doubt, turgor provides the mechanical force for the plastic deformation of growing plant cell walls (Hsiao et al., 1998). However, the rate at which turgor is generated after a turgor-consuming expansion event may be of more relevance to the control of cell elongation than turgor itself (Fricke and Flowers, 1998).

Beyond the elongation zone, turgor was always higher than within it (Fig. 2; compare Fricke et al., 1997; Martre et al., 1999). This suggests that as elongation ceases, walls harden and turgor increases. However, cell volume increases significantly beyond the elongation zone (Fig. 7; compare Fricke and Flowers, 1998). Wall hardening or secondary cell wall formation in grass leaves may proceed in a way that selectively eliminates elongation growth (for an analysis of similar effects in roots, see Liang et al., 1997).

Osmotic and ψ Adjustment during NaCl Stress

At cell and organ level, ψ gradients—the driving force for water uptake—between growing cells and the root medium were maintained during salt stress (Fig. 4). Clipson et al. (1985), using the cell-pressure probe technique, reached the same conclusion for young leaves of the halophyte *Suaeda maritima*.

Elongating cells adjusted osmotically to changes in external ψ by accumulating more solutes (Fig. 3) and by reducing the volume expansion rate (Fig. 1). Hu and Schmidhalter (1998) obtained similar results in wheat. The rate of solute deposition was highest at moderate stress but was similar to the control level at more severe stress (Fig. 8). Thus, at 120 mM NaCl, cells adjusted to changes in external ψ through the largest increase in osmolality (Fig. 2), but achieved this by reduced volume expansion (Fig. 1) rather than by increased solute deposition rates (Fig. 8). This observation can be explained in two ways.

First, the total amount of solutes available for osmotic adjustment may have been limited, reaching maximal deposition rates already at moderate stress level (75 mM NaCl). Sugars and other organic solutes contribute little to osmolality along the elongation zone of NaCl-stressed grass leaves (Hu and Schmidhalter, 1998). Therefore, the rate at which inorganic solutes were supplied to the elongation zone may have limited cell expansion. As a consequence, the rate of cell expansion had to slow down to allow

for maintenance of ψ gradients between elongating cells and the xylem solution.

Second, a limitation in the rate at which solutes were taken up and deposited may have caused cells of 120 mM NaCl plants to grow slowest. It is possible that expanding cells of plants exposed to 120 mM NaCl were metabolically or energetically (Yeo, 1983) limited in their ability to accumulate solutes at rates as high as plants exposed to 75 mM NaCl. If so, cells needed to expand and dilute solute contents at lower rates to maintain ψ gradients and water uptake.

Turgor was changed only slightly in plants subjected to massively increased osmotic pressure in the root medium. Therefore, both of the above scenarios would require that cells either regulated turgor within narrow limits or that measured values of turgor were just above the yield threshold of cell walls. It is noteworthy that this does not imply that cell wall properties of control and NaCl-treated leaf cells were different. Constancy in turgor at altered elongation rates may simply reflect differences in the rate at which turgor was generated after each expansion and dilution event.

Solute Supply to Elongating Leaf Cells at High External NaCl

The above considerations suggest that either supply of solutes to the elongation zone or uptake of solutes by elongating cells limited the rate of leaf elongation, particularly at 120 mM NaCl. Transpiration rates of plants and published values of xylem solute concentrations can be used to estimate the rate of solute import into the growth zone via the xylem. This figure can be compared with the rate of solutes that leaves of NaCl-treated plants needed to deposit to adjust cell ψ.

Using data on total amount of solutes per millimeter of elongation zone (Table II), length of elongation zone (Fig. 1), and velocity of leaf elongation (data for non-pricked plants), bulk-deposition rates of solutes were calculated for the elongation zone. Values were 0.609, 0.721, and 0.532 μmol (growth zone)$^{-1}$ h^{-1} for control plants and plants exposed to 75 and 120 mM NaCl, respectively (Fig. 8A). At the time of analyses, transpirational water loss of leaf three was 212, 141, and 130 μL leaf^{-1} h^{-1} for control, 75 mM NaCl, and 120 mM NaCl plants, respectively (not shown). Thus, to satisfy the osmolyte requirements of the elongation zone, xylem sap at a total solute concentration of 2.9 mM (0.609 μmol 212 μL^{-1}; control), 5.1 mM (75 mM NaCl), and 4.1 mM (120 mM NaCl) was required. These figures are the smallest possible estimates because they do not take into account (a) increased solute demand due to lateral cell expansion of cells beyond the growing zone, (b) the possibility that activity coefficients of (mainly inorganic) cell solutes were <1 (Fricke et al., 1994), (c) supply of solutes, particularly sugars and K^+, to the elongation zone via the phloem (Wolf et al., 1991), and (d) consumption of xylem solutes such as nitrate and phosphate for synthesis of amino acids and macromolecules. Munns (1985) reported that xylem-sap concentrations of Na^+, Cl^-, and K^+ together amounted to about 12 to 16 mM in barley grown at 25 to 150 mM external NaCl. Transpiration rates were higher in Munns' (1985) experiments than in the present study. Considering that xylem sap concentration is inversely related to sap flow (Munns, 1985), it seems unlikely that in the present study, cell elongation in NaCl-treated barley was limited by the rate at which solutes were supplied to the elongation zone. However, it should be noted that xylem solute concentrations required to sustain leaf cell elongation are of the same order of magnitude as measured (Munns, 1985) ones.

Growth-Associated ψ Gradients and Pathways of Water Movement

There can be no doubt that growing cells must have a more negative ψ than the water source—in the present study, leaf xylem. However, controversy exists as to the magnitude of gradients, and as a consequence, the extent to which tissue-hydraulic conductance limits growth (Boyer et al., 1985; Steudle, 1989; Cosgrove, 1993; Nonami et al., 1997).

The magnitude of ψ gradients in the growth zone (Fig. 5) suggests that the hydraulic conductance of the path between leaf xylem and expanding epidermal cell was low and (co-) limiting cell expansion rate, at least in control and mildly stressed plants. Based on the determination of ψ at cell level, and the derivation of xylem ψ, the present study confirms earlier results obtained by Fricke et al. (1997) for barley and Martre et al. (1999) for tall fescue. Both authors supported (or could not rule out) their previous results by subsequent anatomical and theoretical analyses (Fricke and Flowers, 1998; Martre et al., 2001). Fricke and Flowers (1998), studying the elongation zone of the same barley cultivar applied the theory of Molz and Boyer (1974) and Philip (1958) to calculate tissue diffusivities and predict growth-associated ψ gradients. Predicted gradients (-0.18 MPa) were in the range of values reported here for control plants (-0.14 to -0.22 MPa). Fricke and Flowers (1998) based their calculations on the assumption that water moves between leaf xylem and peripheral, elongating epidermal cell along a symplastic or transcellular path. This assumption seems justified (see also the conclusion reached by Boyer [1974] for dicotyledonous leaves). The composite model of water transport developed for the root (Steudle, 2000) predicts that hydrostatic gradients cause water to move along the apoplastic path, whereas osmotic gradients cause water to move along the symplastic or transcellular path; the apoplastic path has a solute reflection coefficient of zero

and does not exert osmotic forces. The grass leaf elongation zone is non-transpiring. It is not known to which degree water potentials of growing cells cause a tension (e.g. soybean; Boyer, 2001) or accumulation of solutes in the apoplast. Hence, at times of (considerable) xylem tension, water movement toward peripheral cells may only be driven by osmotic gradients and proceed along the transcellular or symplastic path. On the contrary, water withdrawal from the elongation zone during periods of increasing transpiration (hydrostatic forces) may occur along the apoplastic path. Suberization (Hattersley and Browning, 1981; Evert et al., 1996) and aquaporin activity (Frangne et al., 2001) in the bundle sheaths may play a key role in regulating this flow.

CONCLUDING REMARKS

Leaf and cell elongation rates in barley exposed to NaCl are not limited by the magnitude of cell turgor. As Cutler et al. (1980) stated, "responses of cell enlargement and leaf elongation to alterations in water status may be described without explicit reference to turgor." If growth limitation in NaCl-treated barley is due to biophysical causes, leaf elongation rate is most likely limited by the rate at which expanding cells can take up solutes from the xylem to maintain ψ gradients toward the external solution. This applies particularly to plants exposed to higher levels (120 mM) of NaCl and is consistent with previous reports on salt-stressed barley (Delane et al., 1982; Munns, 1993). Frensch and Hsiao (1994) reached a similar conclusion for elongating cells of water-stressed maize roots. At moderate stress levels (75 mM NaCl), cell elongation rates in barley leaves may be partly limited by the rate of protoplasmic water supply to cells, similar to the situation in control plants. The apparent paradox that an excess of external solutes (Na^+ and Cl^-) may limit leaf cell expansion through insufficient availability of osmolytes highlights the evolutionary pressure exerted on plants in saline habitats to develop processes for the uptake, transport, and storage of Na^+ and Cl^-, which are compatible with cell function.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Barley (*Hordeum vulgare* L. cv Golf) was grown hydroponically on modified Hoagland solutions (one-half-strength of recipe given by Fricke et al. [1997]) at a photosynthetically active radiation at third-leaf level of 250 to 300 μmol photons m^{-2} s^{-1}. Temperature in the growth facility was partly dependent on weather conditions and ranged from 17°C to 24°C during the day (16 h) and 13°C to 20°C during the night (8 h). Air humidity was not controlled.

Plants were exposed to negligible (1 mM, control), moderate (75 mM), and severe (120 mM) NaCl loads in the root medium. NaCl was added in increments, i.e. 37.5 mM in the evening 2 d before leave three emerged, another 37.5 mM the following morning, and the remaining 45 mM the same day in the evening (120 mM NaCl treatment only). Plants were analyzed after they had been exposed to the final NaCl concentration for 3 to 5 d.

Growth Analysis

Third leaves were analyzed at a developmental stage in which sheath elongation contributed insignificantly to whole-leaf elongation; the ligule was located within 2 to 5 mm from the leaf base. The velocity of elongation of the third leaf was measured in undisturbed plants and in plants mounted on the probe stage. In undisturbed plants, leaf elongation velocity was calculated from leaf lengths measured daily twice. In plants on the probe stage, leaf elongation was determined after completion of single-cell analysis (which took between 10 and 30 min). An ink mark was placed on the mature leaf blade, and its displacement was followed under a stereomicroscope for up to 1 h.

The profile of leaf elongation growth was determined in pin-prick experiments (Schnyder et al., 1987). The basal portion of the leaf sheaths of the plants was pricked with entomological needles (0.2 mm in diameter) at approximately 4-mm intervals, and the displacement of holes was recorded in the third leaf after 6 to 7 h following dissection of the plant. Segmental growth was quantified as a relative growth rate (Green, 1976; Hunt, 1982), termed relative elemental growth rate, REGR:

$$\mathrm{REGR} = (\ln L_f - \ln L_i)(\Delta t)^{-1} \quad (1)$$

where L_i and L_f denote the initial and final segment length, respectively, and Δt stands for the duration of the experiment. Following the theoretical considerations discussed by Peters and Bernstein (1997), REGR was plotted versus average segment position to yield profiles of relative elemental elongation rates. To better visualize the idealized shape of the growth profiles, running means of nine consecutive data points were plotted with the original data.

Pricking reduced the velocity of leaf elongation by between 59% and 66%. The REGR profiles were corrected for this reduction, assuming that the reduction was spread proportionally along the elongation zone (Schnyder et al., 1987; Hu and Schmidhalter, 2000).

Analysis of Bulk-Leaf Extracts

Osmolality

Plants were placed on moistened tissue paper and the first two leaves were removed. The basal region of leaf three was sectioned into three segments (0–25, 25–50, and 50–60 mm above the base). Younger leaves were removed, and segments were placed in custom-built tubular inserts in 1.5-mL microcentrifuge tubes. The basal opening of the inserts was covered by fine gauze, allowing cell sap but not tissue fragments to pass. The sample was frozen in liquid nitrogen and thawed (two cycles) and then spun for 3 min at 11,600*g* in a microcentrifuge (Micro-Centaur, MSE, Loughborough, UK). Samples (about 10 μL) were collected

and stored under a layer of water-saturated liquid paraffin in 0.5-mL centrifuge tubes. Small aliquots of samples were analyzed for osmolality using a picoliter osmometer (Bangor University, Bangor, UK) as previously described for single-cell samples (Malone et al., 1989). Samples kept overnight in a freezer gave similar results as those analyzed immediately after sampling.

Water Contents

Leaves sectioned as described above were placed into preweighed 1.5-mL microcentrifuge tubes. Tubes were weighed again to determine fresh weights. Dry weights were obtained after drying samples for 2 d at 55°C. Water contents per millimeter leaf length were calculated from fresh and dry weight data.

Single-Cell Analyses

Turgor Pressure

Turgor was measured in epidermal cells using the cell-pressure probe technique (Steudle, 1993; Tomos and Leigh, 1999). Cells were located either along the leaf base (20, 40, and 60 mm from the leaf insertion) or in the center of the emerged blade. Three alternative methods were applied to access the basal leaf region.

Method I

A plant was taken from the nutrient solution, and the seed hull was removed. The coleoptile and first and second leaves were excised, and the cut surface was sealed with a thin film of Vaseline. Care was taken to avoid damaging leaf two while peeling it off and to prevent leakage of cell sap onto leaf three. To support the exposed leaf base, the plant was placed horizontally on one-half of a longitudinally split plastic tube that was lined with tissue paper soaked in distilled water. Roots were kept in nutrient solution from the pot the plant had grown in, and the base of leaf three was covered with tissue paper moistened with distilled water. The plant was left on the stage for 15 to 20 min before analyses. Turgor was measured at 20, 40, and 60 mm from the leaf base. At each location, a small piece of moist tissue paper was removed to allow access to the cells and was put back after completion of measurement. The sequence of analysis along the leaf base, i.e. upwards from 20 to 60 mm or vice versa, had no effect on turgor values obtained.

Method II

Plants were prepared and analyzed in the way described above (method I), except that the tissue paper used to line and cover the exposed basal leaf region was moistened with NaCl solution of the concentration prevailing in the root medium. Because nutrient media of control plants contained only 1 mM NaCl, control plants were not analyzed according to method II.

Method III

The seed hull, coleoptile, and first leaf were removed, leaving leaf two in its position covering the base of leaf three. A small window was cut into the sheath of leaf two at 20 to 24 mm above the base of leaf three under a stereomicroscope. Plants in which leaf three was damaged during this process were discarded. The window was sealed with Vaseline, and a piece of clingfilm was placed on top of it. The plant was put back into its pot, and the weakened leaf base was supported with an extra piece of foam rubber. After 4 to 5 h, the plant was mounted on the probe stage with roots kept in nutrient solution. The clingfilm (but not Vaseline) was removed. Turgor measurements commenced after 15 to 20 min. Leaf elongation velocity in method III plants was measured after preparation but before transfer to the probe stage and again after completion of turgor measurements.

Osmolality Measurements

Single-cell osmolality was determined by picoliter osmometery as detailed previously (Malone et al., 1989). Plants were prepared according to methods I and II (see above). In each plant, two to three cell sap samples were extracted at 10, 20, 30, 40, and 60 mm above the leaf base, which took 10 to 15 min. Each sample was placed immediately under a drop of water-saturated liquid paraffin on the osmometer stage. Osmolality was determined for all samples and NaCl standards (0, 100, 200, and 400 mM) in the same freezing cycle.

Solute Deposition Rates

Solute deposition rates were calculated from leaf elongation data of undisturbed plants, bulk-tissue osmolality, and bulk-tissue water contents. To convert mosmoles per kilogram into micromolar, it was assumed that 1 kg of cell sap approximated 1 L and that cell sap behaved like an ideal solution.

Cell Lengths

Cell length was determined for cells located adjacent to stomatal rows in the adaxial epidermis of leaf three. A double-replica technique was used (Fricke et al., 1997) to obtain positive impressions from cells in the emerged part of the blade, at positions that had passed 2 d earlier through the region at 50 to 60 mm from the leaf base (positions were traced back based on total leaf elongation during that period).

Transpiration Rates in Leaf Three

Transpiration of leaf three was determined by covering the blades of the first two leaves with Vaseline and measuring the weight decrease (water loss) over 8 h in a culture pot carrying three plants of the same age.

Statistics

Statistical significance of differences between data sets was evaluated by Student's *t* test or, where possible, by paired *t* test. When overall means were calculated from the means of individual data sets, Gauss' law of error propagation was used to calculate the SD of the overall mean from the SDs of the individual data sets.

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